Video Article

Characterization of Thymic Settling Progenitors in the Mouse Embryo Using *In Vivo* and *In Vitro* Assays

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Abstract

Characterizing thymic settling progenitors is important to understand the pre-thymic stages of T cell development, essential to devise strategies for T cell replacement in lymphopenic patients. We studied thymic settling progenitors from murine embryonic day 13 and 18 thymi by two complementary *in vitro* and *in vivo* techniques, both based on the “hanging drop” method. This method allowed colonizing irradiated fetal thymic lobes with E13 and/or E18 thymic progenitors distinguished by CD45 allotypic markers and thus following their progeny. Colonization with mixed populations allows analyzing cell autonomous differences in biologic properties of the progenitors while colonization with either population removes possible competitive selective pressures. The colonized thymic lobes can also be grafted in immunodeficient male recipient mice allowing the analysis of the mature T cell progeny *in vivo*, such as population dynamics of the peripheral immune system and colonization of different tissues and organs. Fetal thymic organ cultures revealed that E13 progenitors developed rapidly into all mature $CD3^+$ cells and gave rise to the canonical γδ T cell subset, known as dendritic epithelial T cells. In comparison, E18 progenitors have a delayed differentiation and were unable to generate dendritic epithelial T cells. The monitoring of peripheral blood of thymus-grafted $CD3^{-/-}$ mice further showed that E18 thymic settling progenitors generate, with time, larger numbers of mature T cells than their E13 counterparts, a feature that could not be appreciated in the short term fetal thymic organ cultures.

Video Link

The video component of this article can be found at http://www.jove.com/video/52795/

Introduction

T lymphocytes, bearing the αβ or the γδ T cell receptor (TcR), differentiate in a specialized organ, the thymus. The fully developed thymus is organized into two distinct regions: the cortex, where thymic progenitors develop and where thymocytes that productively rearrange the TcR β and α chain genes are rescued from programmed death (a process known as positive selection); and the medulla, where selected thymocytes with too strong reactivity to self-ligands are deleted (negative selection)[1,2]. The thymus originates from the endodermal layer of the third pharyngeal pouch that is later surrounded by mesenchymal cells[3]. It is colonized by hematopoietic progenitors starting at embryonic day E12 and, thereafter, continuous recruitment is required for normal T cell development[4]. Thymic immigrants evolve through successive developmental stages, orchestrated by a tightly regulated program, initiated and maintained by the activation of the Notch signaling pathway on thymocytes upon interaction with its ligand, delta like 4, expressed on thymic epithelial cells (TECs)[5].

Thymocyte development starts at the so-called $CD4^-CD8^-$ double negative (DN) stages. DN thymocytes can be further subdivided according to the expression of CD25 and CD44 into DN1 ($CD25^-CD44^+$), DN2 ($CD25^+CD44^+$), DN3 ($CD25^+CD44^-$) and DN4 ($CD25^-CD44^-$). CD24 (HSA) and CD117 (c-Kit) further subdivides the DN1 compartment into 5 subsets where DN1a and b correspond to early thymic progenitors (ETP). Thymocytes rearrange the TcR δ, β and α chains at the DN stage and undergo pre-TcR selection (DN3-DN4 stages). They further transit to the $CD4^+CD8^+$ double positive (DP) compartment where the TcR α chain rearranges prior to positive and negative selection. At this stage most thymocytes are eliminated and only a small percentage (3-5%) reach the $CD4^+$ or $CD8^+$ mature T cell compartment.

The lymphoid differentiation pathway progresses through the stages of HSCs that generate multipotent progenitors (MPP) and lymphoid-primed multipotent progenitors (LMPP) that lost the erythrocyte and megakaryocyte potential[6]. LMPP are phenotypically defined by the absence of differentiated blood cell markers (lineage negative, Lin^-), the expression of c-Kit (CD117), Sca-1 and Flt3/Flk2 (CD135) and the absence of detectable levels of the interleukin (IL)-7 receptor α chain (IL-7rα or CD127). LMPPs further differentiate into common lymphoid progenitors (CLP)[7] that by that stage have lost the capacity to generate myeloid cells. CLP retain lymphocyte (B and T cell), NK cell, DC and innate lymphoid cell (ILC) potential, and differ from LMPP by the expression of CD127 and the absence of high levels of Sca-1.

Although the nature of the thymic settling progenitors (TSP) has been extensively debated[8], it became recently clear that TSP change phenotype, differentiation potential and function, throughout development[9]. We performed *in vitro* and *in vivo* assays to characterize the TSP, isolated by FACS cell sorting from either E13 (first wave) or E18 (second wave). Fetal thymic organ cultures (FTOC) with irradiated thymic lobes colonized by equal numbers of E13 and E18 progenitors, bearing different allotypic markers, allowed following their progeny in a similar developmental environment and revealed cell intrinsic properties, different between both types of progenitors. Thymic lobes colonized by either E13 or E18 TSP allowed development without selection due to competition between both progenitors. *In vivo* transplantation of the colonized thymic lobes further showed that also the mature progeny of E13 and E18 TSP have different biologic properties *in vivo*. TSPs from the first wave rapidly generate T cells but give rise to low numbers of αβ and γδ T cells. Among the latter we detected Vγ5Vδ1 dendritic epithelial T cells (DETC), that have an invariant TcR, migrate to the epidermis where they exert a function in wound healing and are only produced during embryonic development[10]. In contrast, TSP from the second wave take longer time to generate high numbers of TcR^+ T cells and are unable to generate DETC.

Protocol

Ethics statement: all experiments were performed according to the Pasteur Institute Ethic Charter, approved by the French Agriculture Ministry, and to the EU guidelines. A manipulator with training on small rodent surgery, certified by the French Ministry of Agriculture, performs all surgical interventions.
NOTE: See in annex **Table 1** showing the 5-step plan procedure.

1. Selection of the Embryos

1. Use 36 C57BL/6 CD45.2 females, 12 females CD45.1, 12 C57BL/6 CD45.1 males and 12 C57BL/6 CD45.2 males. Crossing the females with either male genotype will produce embryos CD45.1/2 and CD45.1 used as a source of TSP or CD45.2 used as a source of recipient thymic lobes. House all males individually.
2. To obtain embryos for E18 TSP isolation, place 2 females in a cage with one male CD45.1 at 6 pm, 21 days before the grafting experiment. Check the presence of a vaginal plug the next day, before noon. Separate the plugged females.
3. To obtain E14 embryos to be colonized by TSP, repeat the procedure above (1.2), using males CD45.2, 17 days before the grafting experiment.
4. To obtain embryos to isolate E13 TSP, repeat the procedure above (1.2), using males C57BL/6 CD45.1, 16 days before the grafting experiment.

2. Dissection of the Embryos Under a Horizontal Laminar Flow Hood

NOTE: Two days before the grafting experiment.

1. Sacrifice the pregnant females by cervical dislocation or by progressive administration of CO_2 gas during at least 5 min, in a saturating closed chamber. Ensure death by definitive arrest of cardio-respiratory movements. Wet the abdomen with 70% ethanol.
2. Make a longitudinal incision in the skin at the midline abdomen and open it by pulling the skin apart. Open the peritoneum with forceps and scissors without touching the digestive tract. Pull out the bifid uterus and separate it from the vagina with the scissors.
3. Place the uterus in a 90 x 15 mm Petri dish containing 40-50 ml DPBS and cut transversally between each embryo's decidua.
4. With the pair of fine tip scissors and a fine forceps remove the muscle membrane of the uterus, take out the embryos by cutting between the placenta and the yolk sac, and remove the amnios, which is retaining the embryos.
5. Place the embryos in a Petri dish 90 x 15 mm containing HBSS + 1% fetal calf serum (FCS). For embryos older than E15, remove the heads with a pair of scissors before any further dissection.

3. Isolation of the Thymus

1. Under the binocular magnifying lens, place the embryo, lying in supine position (ventral view), on a wet gauze bedding.
2. Insert one forceps longitudinally along the cartilage of the sternum, pinch and open the thoracic grid. With curved forceps, pull the thoracic wall aside to visualize the thymic lobes on each side of the trachea.
3. Carefully hold each lobe by pinching underneath with a fine forceps and place them in a Petri dish containing 1 ml HBSS + 1% FCS. Isolate the lobes and remove surrounding connective tissue with two 1 ml syringes + 26 G ⅜" needles, without damaging the capsule.
4. Wash the lobes in 3 ml of HBSS + 1% FCS to eliminate blood cells.
   NOTE: Before E15, thymic lobes are located on each side of the trachea and later fuse in the middle to constitute a bi-lobar organ.

4. Cell Suspensions

1. Tease apart the lobes under the binocular magnifying lens, with two 26 G needles mounted on 1 ml syringes, in HBSS + 1% FCS. Filter the cell suspension through a nylon mesh.

5. Staining with Fluorescent Antibodies and Cell Sorting

NOTE: All antibodies are previously titrated to obtain optimal definition of the populations (the titration can vary with the antibody batch).

1. Incubate the thymocytes (E13 or E18) for 15-30 min at 4 °C with a 100 µl of a cocktail of biotinylated antibodies to stain lineage positive cells (anti-CD19, anti-Gr1, anti-Ter119, anti-NK1.1, anti- CD11c, anti-CD3, anti-CD4, anti-CD8, anti-CD25).

2. To wash away excess of antibodies spin down the tubes with 4 ml HBSS + 1% FCS at 280 x g for 7 min, eliminate the supernatant and re-suspending the pellet in fresh HBSS + 1% FCS. Repeat the centrifugation.
3. Incubate the E18 biotin-labeled cells with streptavidin microbeads during 15 min at 4 °C and wash the cells twice in HBSS 1% FCS. Re-suspend the cells in 2 ml HBSS + 1% FCS. Most E13 thymocytes are lineage negative and therefore do not need MACS enrichment prior to cell sorting.
4. Pass the cells on the LS column, according to the manufacturer's instructions. When all the cell suspension entered the column add 2 ml HBSS + 1% FCS. Recover the 4 ml of the column flow through and centrifuge the cells for 7 min at 280 x g.
5. Re-suspend the pellet of either depleted E18 or total E13 thymocytes in 50 µl of the TSP antibody mix (anti-CD117 APC, anti-CD135 PE, anti-CD127 PECy7, anti-CD24 FITC, anti-CD44 APCCy7 and streptavidin Pacific Blue) and incubate for 20 min at 4 °C in the dark.
6. Wash the cells with 4 ml HBSS + 1% FCS and re-suspend the cells in 1 ml HBSS + 1% FCS with propidium iodide.
7. Sort the TSPs $Lin^{-}CD44^{+}CD117^{+}CD24^{low}CD127^{+}CD135^{+}$ cells in a FACS (with 4 lasers) onto a 1.5 ml tubes containing 200 µl complete medium + 20% FCS. Gate first the cells according to their size and granularity on the FSC and SSC channels. Eliminate dead cells (staining with propidium iodide), gate out doublets and score the fluorescence in exponential scales. Around 100 or 600 TSPs can be obtained from one E13 or one E18 thymus, respectively.

6. Colonization of E14 Thymic Lobes with Progenitors: Hanging Drop Technique

1. Irradiate (30 Grays = 30 Gy) E14 thymic lobes from CD45.2 embryos, 3 hr before culture.
   NOTE: E14 or E15 thymic lobes have been successfully used as recipient of T cell progenitors. Using recipient thymic lobes of later gestational days results in premature necrosis due to the bigger size of the organ while thymic lobes at earlier gestational days develop poorly, probably due to incomplete development of the epithelial cells.
2. Centrifuge sorted TSPs and re-suspend the cells in culture medium (OPTI-MEM complete medium with 10% FCS, penicillin (50 units/ml), streptomycin (50 µg/ml) and 2β-mercaptoethanol (50 µM)) such that 35 µl contain 500 TSP.
3. Place a 35 µl drop of cell suspension every other well of a 60 well Terasaki plate.
   NOTE: 35 µl drops can fuse if they are placed in contiguous wells.
4. Place one irradiated lobe on the surface of each drop. Close the Terasaki plate and turn the plate upside down to let the cells reach the apical pole of the drop by gravity.
5. Hit gently the upper side of the inverted plate to force the lobes to slide to the apical edge of the drop. Incubate the inverted Terasaki plate in a humidified incubator for 48 hr at 37 °C + 5% CO_2. After colonization, either culture E14 thymi in FTOC[7], or graft under the kidney capsule of a recipient adult mouse [8].

7. Fetal Thymic Organ Culture

1. Place 3 ml of complete medium on a 35 mm Petri dish. Place an isopore membrane filter floating on the medium. Place the reconstituted lobes on the edge of the membrane with a forceps (no more than 8 lobes on one membrane).
2. Place the Petri dishes containing the thymic lobes inside a 90 mm Petri dish, together with a 35 mm dish, without cover, containing 2 ml of water, to ensure optimal humidity. Incubate 12 days at 37 °C + 5% CO_2.

8. Grafts Under the Kidney Capsule Under Sterile Conditions

NOTE: The kidney parenchyma is surrounded by connective tissue forming a capsule. The sub-capsular region is particularly rich in blood and lymphatic vessels thereby providing a suitable environment for the development of grafts (*e.g.* thymic lobes, pancreatic islets or newborn hearts). Grafts are usually done on the left kidney because it is more accessible than the right kidney. $CD3^{-/-}$ male mice were used as recipients thus avoiding graft versus host reaction due to minor histocompatibility antigens linked to the Y chromosome because sex determination before E15 is not easily done.

1. Sterilize the instruments and wear sterile gloves along the surgery. Anesthetize the mouse by intra peritoneal injection of a solution of Ketamine 10 mg/ml + Xylazine 1 mg/ml diluted in PBS: (50 to 100 µl per 10 g body weight), using a 1 ml syringe. Verify anesthesia by checking lack of interdigital reflexes. Place a drop of hydro-Optimune gel on each eye to prevent dryness during anesthesia.
   NOTE: The solution should be prepared extemporaneously and warmed at room temperature before injection. The anesthesia lasts at least 20 min. The degree of anesthesia should be controlled all along the surgery. The anesthesia can be prolonged by intra peritoneal injection of half dose every 20 min.
2. Place the mouse on its right side, under a horizontal lamina flow hood. Sterilize the surgical field with 70% ethanol and 10% iodide dermic solution. With a forceps part the mouse hair along a 2 cm length just above the joint of the hind leg. Shave the surgical area.
3. With a scalpel, make an incision 1.5 cm length of first, the cutaneous tissue, then with scissors cut the muscular tissue. Apply a slight pressure to both sides of the incision, which will force the kidney out of the abdominal cavity.
4. Apply saline with a cotton-bud to keep the kidney moist. If you have difficulties in exposing the organ, use a flat forceps to pull out the surrounding adipocyte tissue to which the kidney is attached. Maintain the kidney out of the retroperitoneal cavity by a cotton bud placed underneath the organ.
5. With two fine forceps, make a 2-3 mm hole in the capsule (avoid touching the parenchyma to prevent bleeding). During the whole surgical procedure keep the exposed kidney capsule continuously moistened.
6. Insert carefully the lobes under the kidney capsule, by maintaining the wall of the capsule opened and slide the graft under the capsule towards the edge pole. Place up to 6 lobes per kidney.
7. Replace the kidney in the retro-peritoneal cavity. Suture the muscle aponeuroses, then the skin with individual surgeon knots. Wet the wound with 10% iodide povidone solution. Place the mouse on a heated pad at 37 °C.
8. Survey the transplanted mouse until full recovery from anesthesia by control of cardiac, respiratory and whisker movements, mucosal colors until total recovery seen by self contained movements.

NOTE: We recommend injection of analgesic solution (Buprenorphin, 0.1 mg/kg) intra muscle just after the surgery and the 2 days following surgery to prevent post-surgery pain.

9. Keep the operated animal in isolation until fully recovered. Inspect every other day the stitches of the wound to verify and prevent infection. Never observed wound infection following this procedure.
   NOTE: Weekly analysis of peripheral blood cells from the $CD3^{-/-}$ grafted mice allow us to detect the thymic derived populations originated from either E13 or E18 progenitors using the CD45 allotypic marker and T cell lineage specific antibodies (**Figure 3**).

9. Analysis of the TSP progeny by Flow Cytometry

1. Tease the FTOCs individually to obtain single cell suspensions (see protocol section 4).
2. Stain cell suspensions from individual lobes with an antibody mixture containing antibodies recognizing CD45.1 PECy7, CD45.2 AmCyan, CD4 APCCy7, CD8 APC, CD3 Pacific Blue, Vγ5 FITC, Vδ1 PE and incubate as described in section 5.
3. Re-suspend the cells in HBSS + 1% FCS + propidium iodide and analyze in a flow cytometer (see results in **Figure 2**).

Representative Results

In order to choose a method to deplete thymic lobes of endogenous thymocytes allowing the best development of colonizing progenitors, we compared the levels of T cell reconstitution in thymic lobes colonized after irradiation or a 5-day deoxy-guanosine (d-Gua) treatment. The results show that while there is no difference at day 9 of culture, irradiated lobes contained more T cells than those treated with d-Gua, at day 12. Thus, irradiation is more appropriate than d-Gua treatment to obtain T cell development after thymic colonization (**Figure 1**). To study the developmental potential of E13 and E18 TSP, we colonized E14 irradiated thymic lobes with a mixture of equal numbers of the two types of progenitors. The results show that E13 TSPs give rise to less thymocytes and less DP than E18 TSP but, in contrast, E13 TSP generate DETC and higher frequencies of $CD3^{+}$ mature cells (**Figure 2**). To analyze the *in vivo* potential of E13 and E18 TSP, thymic lobes colonized with each type of progenitors were grafted under the kidney capsule of $CD3^{-/-}$ recipients. The results show that, consistent with the FTOC, E13 TSP gave rise to T cells faster than E18 progenitors but the number of circulating T cells was significantly lower (**Figure 3**).

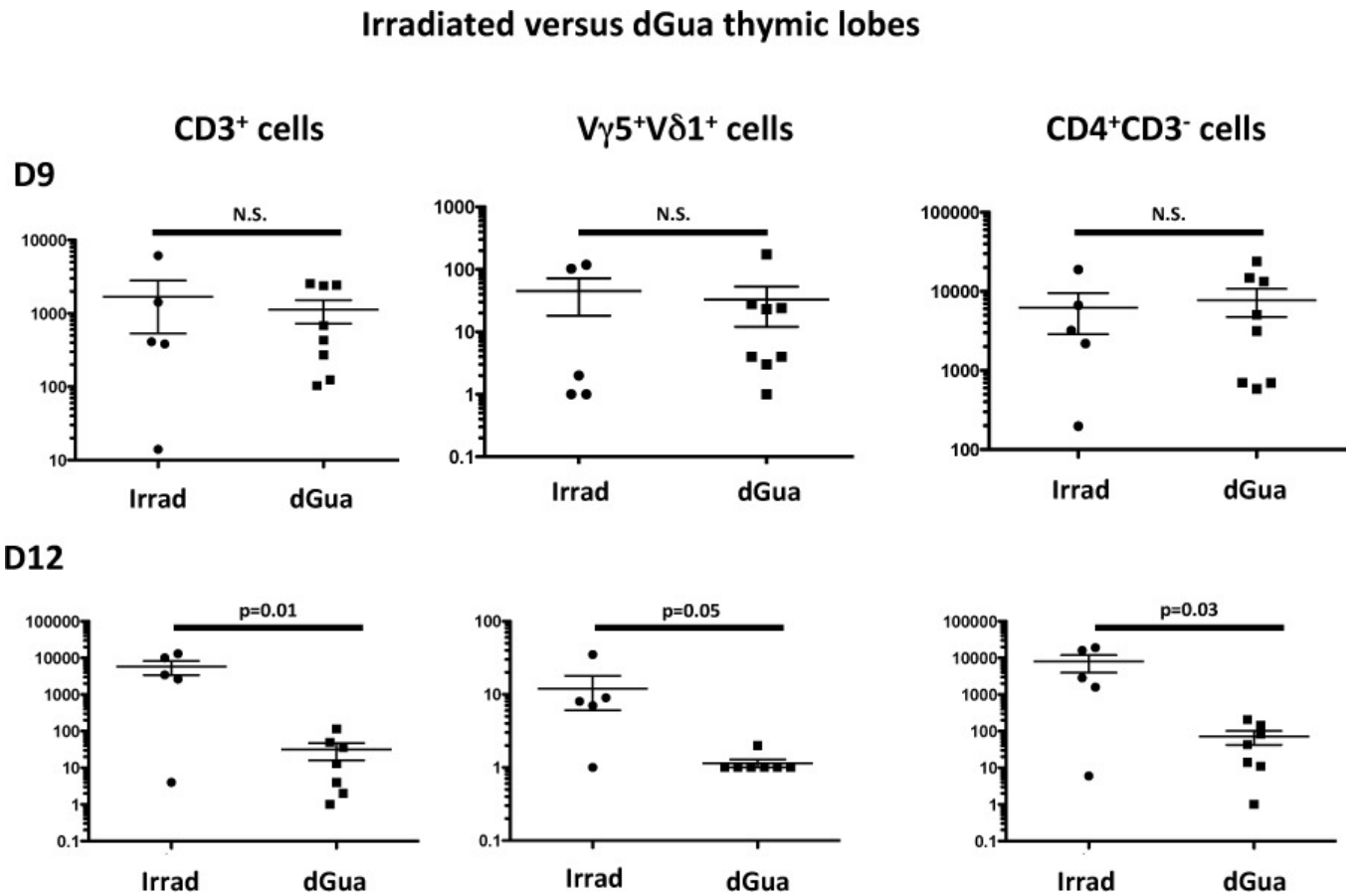

Figure 1: T cell development is more efficient in irradiated than in deoxyguanosine treated, colonized thymic lobes. E14 thymic lobes (CD45.2) were either irradiated with 30 Gy or treated for 5 days with deoxy-guanosine (d-Gua). Thymic lobes were then colonized with 1000 $Lin^{-}CD117^{+}Sca\text{-}1^{+}$ (LSK) E14 FL cells isolated from CD45.1/2 embryos, in hanging drop for 48 hr and cultured on a filter. No differences were observed in the efficiency of endogenous thymocyte depletion between the two groups of thymic lobes. Developing thymocytes stained with antibodies against CD45.1, CD45.2, CD3, Vγ5, Vδ1 and CD4 were analyzed at day 9 and 12 by FACS. Please click here to view a larger version of this figure.

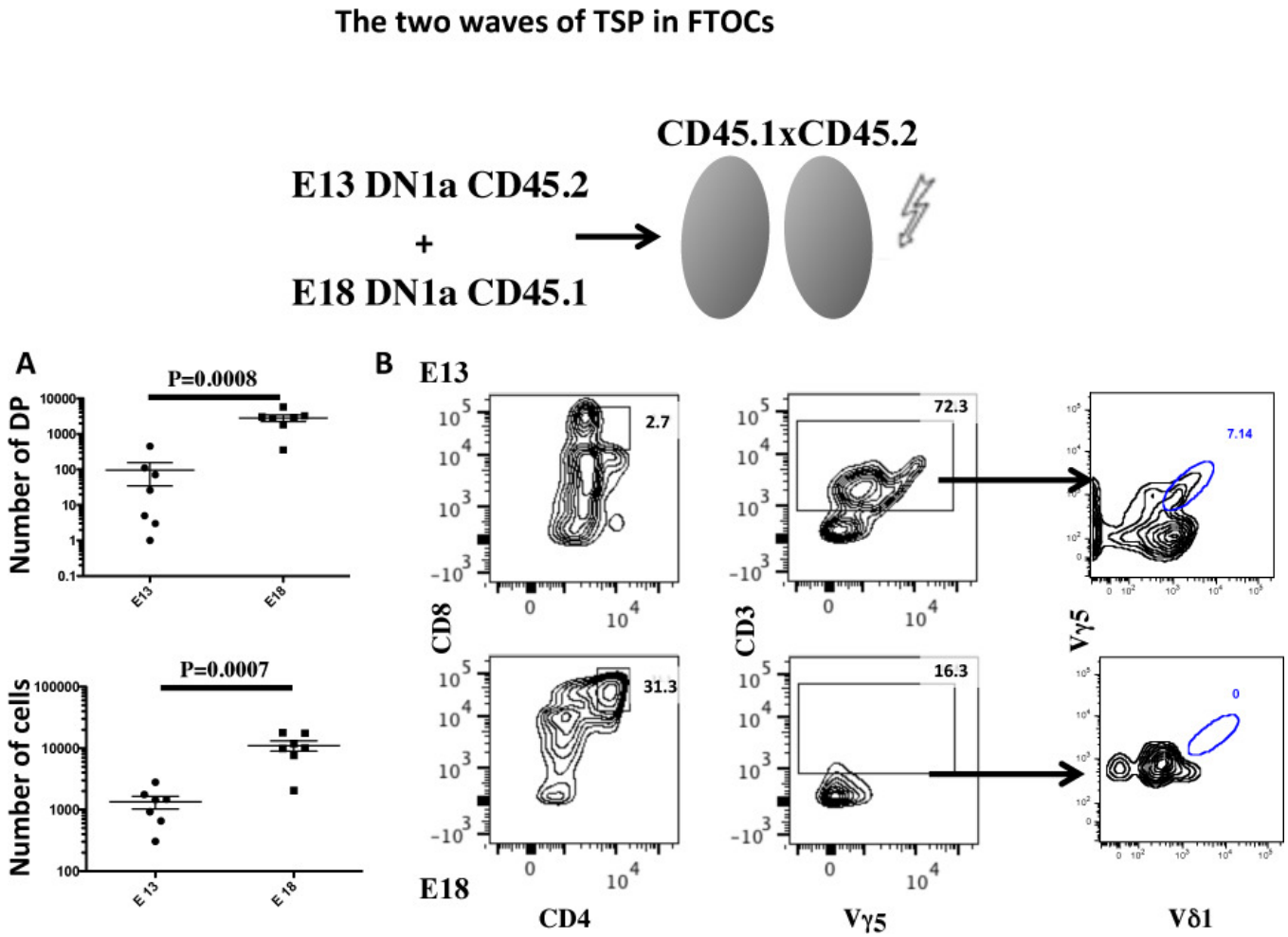

Figure 2: In contrast to E18, E13 TSPs generate Vγ5Vδ1 DETC. CD45.2 thymic lobes were irradiated and colonized for 48 hr with a mixed cohort of 500 E13 TSP from CD45.1/2 embryos and 500 E18 TSP from CD45.1 embryos. Under these experimental conditions, E13 and E18 TSP develop in the same environment and differences in the rate of differentiation and mature T cell subsets observed after culture can only reflect differences in cell intrinsic biologic properties. After 12 days in culture thymocytes from individual lobes were analyzed by flow cytometry after staining with antibodies recognizing CD45.1, CD45.2, CD4, CD8, CD3, Vγ5, Vδ1. (**A**) Panels show the numbers of cells recovered in each lobe. (**B**) Representative flow cytometry profiles. Please click here to view a larger version of this figure.

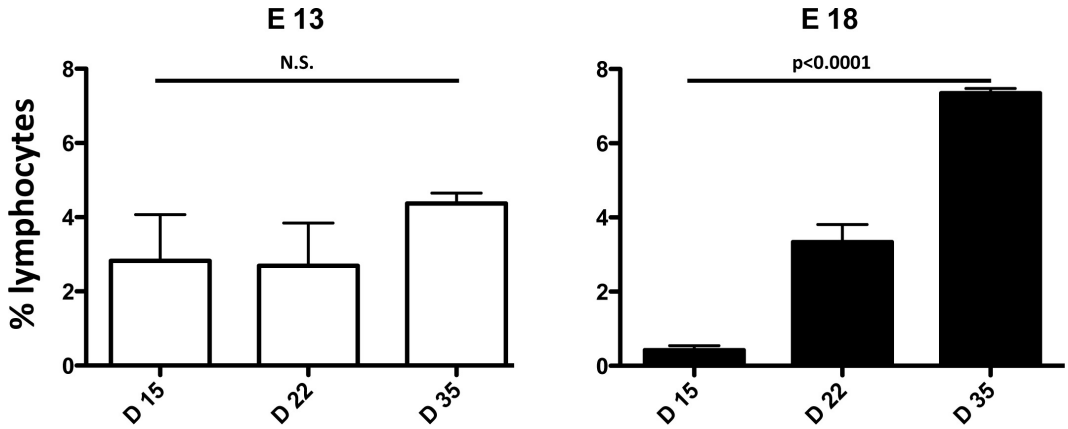

Figure 3: E13 TSPs develop faster than E18 TSPs. CD45.2 thymic lobes were irradiated and colonized for 48 hr with 500 E13 TSP or 500 E18 TSP from CD45.1 embryos. $CD3^{-/-}$ mice were grafted with 4 thymic lobes colonized with either E13 or E18 TSPs. Peripheral blood was collected at weekly intervals and analyzed by FACS for donor (CD45.1) $CD3^{+}$ T cells. Please click here to view a larger version of this figure.

step 1	Mating mice to obtain E18 embryos (day -21); mating mice to obtain E14 embryos (day -17); mating mice to obtain E13 embryos (day -16)
step 2a	Dissection of the embryos, day -2
step 2b	Irradiation of E14 thymic lobes, day -2
step 2c	Preparing, staining, and sorting cells from E13 and E18 thymic lobes, day -2
step 2d	Hanging drop culture, day -2
step 3a	Fetal Thymic Organ Culture, day 0
step 3b	Graft under the kidney capsule, day 0
step 4	FTOC: flow cytometry analysis, day 12
step 5	Graft: weekly flow cytometry analysis of circulating T cells, days 15, 22, 35

Table 1: The 5-step procedure followed in the experiment. Time-table of the experiment from the mating of the different mouse strains up to the analysis of the grafted mice. Grafts are performed in 7 week old mice. Taking the time of transplantation as day 0, day -21 is the mating of mice to obtain E18 embryos, day -17 to obtain E14 embryos, day -16 to obtain E13 embryos, and in day -2 the sorting of E13 and E18 TSP, irradiation thymic lobe and hanging drop technique.

Antibody	Clone Number		Antibody	Clone Number
CD25	7D4		CD19	6D5
CD44	IM7		Ter 119	TER-119
CD24	M1/69		NK1.1	PK 136
CD117	2B8		CD11c	HL3
CD3	145-2C11		Gr1	RB6-8C5
CD4	RM4-5		GD	eBioGL3
CD8	53-6.7		Sca-1	D7
CD135	A2F10		CD45.2	104
CD127	A7R34		Ly5.1	A20

Table 2: Antibodies and clone numbers.

Discussion

Two main assays can be used to analyze T cell differentiation *ex vivo*. The most recently reported is the co-culture of hematopoietic progenitors with BM stromal cells, OP9, expressing the ligands of Noth1, delta like 1 or 4[12]. This 2-D assay is easy to perform, highly efficient and sensitive, allowing analysis at the single cell level. However, it neither supports T cell development beyond the stage of DP nor the generation of γδ DETC[13], both of which require direct interactions with the thymic epithelium.

FTOCs have long been used to analyze T cell development[3]. The major strength of this assay is allowing the generation of all mature T cell compartments, a property granted by the efficient 3-D interactions of thymocytes with the thymic epithelium. We tested here two methods currently used to deplete endogenous thymocytes thus allowing efficient colonization by exogenous progenitors. Both ionizing irradiation and deoxyguanosine treatment efficiently depleted developing thymocytes. However, only irradiated thymic lobes sustained a robust T cell development for longer periods of time suggesting that d-Gua treatment might also affect components of the epithelial compartment. Embryonic thymic lobes were colonized by exogenous progenitors using the “hanging drop” method. Colonization of irradiated lobes with both populations in competition allows detecting different cell autonomous biologic properties. Colonization with only one of the TSP subsets reveals their differentiation capacity in a non-competitive environment.

The disadvantage of this method is a lower efficiency in the frequency of TSP that develop into T cells as compared to OP9Dl co-cultures[14]. However we have done single DN1 or DN2 cells to colonize individual thymic lobes with efficiency closer to that obtained with the stromal cells. A ten-fold reduction in efficiency of T cell production is found when FL or BM hematopoietic progenitors are used for the FTOC that is not observed in the OP9 delta like cultures. This less efficient development suggests that not all BM or FL cells with T cell potential can colonize the thymus.

Grafting colonized thymic lobes offers a better nutrient and oxygen supply to developing thymi than in FTOC, and the possibility to follow the fate of the progeny of TSP, *in vivo*. Using these two combined strategies we could describe the steps of differentiation and functional potential of the progeny of TSPs. Because $CD3^{-/-}$ mice CD45.2[15], that cannot develop mature T cells, were used as hosts, we could together with the CD45 allotypic differences, unambiguously follow the newly generated T cells in their natural environments

The novel aspects of the experimental procedure described here is the combination of reconstituted FTOC with *in vivo* transplantation. This allowed identifying and tracing the progeny of defined subsets of hematopoietic progenitors. We found that TSP from the first and second waves generate different subsets of γδT cells, different numbers of αβT cells and have different kinetics of differentiation.

Stage of the embryos: the day 0 is considered 18 hr after the mating of the mice, according to the plug detection. Dissections of the thymi requires a good cold light 150 W, a binocular dissection microscope and some practice for dissections, anesthesia and graft procedures.

Disclosures

The authors declare that they have no competing financial interests.

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